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(54) Abstract Title
**Animal feed**

(57) Animal feeds comprising a compound that produces an antimicrobial substance and a compound that disrupts the phospholipid layer of bacteria are disclosed. The compounds can act synergistically against bacteria, and therefore improve the growth or the feed conversion ratio of animals. The feed may be fed to monogastric and/or non-ruminant animals such as poultry, pigs, piglets, (veal) calves or fish. The compound producing an antimicrobial substance can be an enzyme such as an oxidase (e.g. glucose oxidase), peroxidase, lipoxygenase, synthase or phosphorylase and can generate a toxic substance such as phosphatidyl choline, hydrogen peroxide, HOCl, OSCN or xylitol-5-phosphate. The phospholipid disrupting compound can be a phospholipase, polyunsaturated fatty acid (PUFA, such as arachidonic acid) or a chelating agent (such as EDTA).

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# ANIMAL FEED

## Field of the Invention

The present application relates to animal feeds, or additives or premixes therefor, that contain a compound that can produce a (toxic) antimicrobial substance and a compound that can disrupt the (inner and/or outer) phospholipid layer of bacteria. These can act synergistically as antimicrobial agents.

## Background of the Invention

Monogastric animals such as pigs, poultry, veal calves and fish are grown intensively for the production of meat, fish and eggs. These animals are fed diets containing a variety of raw materials of animal and/or vegetable origin to supply energy and protein. Most of the feed that is consumed is produced commercially, but a significant part is produced on the farm and fed directly. The feed is often supplemented with vitamins and minerals to meet the animal's nutrient requirements. The use of industrially produced enzymes in these feeds has now almost become common practice. Enzymes include phytases, amylases, proteases, glucanases, endoxylanases and mannanases. however, feed costs are the most important cost factor in animal production.

These enzymes are used to promote growth and feed conversion, and to reduce the environmental pollution produced by manure from pigs, poultry and fish. However, feed costs are the most important cost factor in animal production. While antibiotics have been routinely added to animal feed, it has been reported that human pathogenic bacteria could develop a resistance against those antibiotics or antibiotics related to those. This has made it more difficult to cure people from bacterial infections, and the widespread use of antibiotics in animal feed has been blamed by various experts in the acceleration of build-up resistance to various antibiotics. This has led to a ban on the use of most antibiotics as growth promoters in animal feed in the European Union. It is likely that other countries will follow this example due to pressure from consumer and healthcare organisations. The feed industry is therefore more interested in natural additives with growth promoting effects, without any therapeutic side effects in humans.

WO-A-00/21381 (DSM N.V.) teaches animal feeds which contain at least two antimicrobial enzymes and a polyunsaturated fatty acid (PUFA). One of these enzymes can be lysozyme. At that time it was not realised that a PUFA could disrupt the outer phospholipid layer of bacteria, and while it was known that the PUFA was beneficial, its mechanism was unknown, and therefore its use as a compound for disrupting the outer phospholipid layer was not disclosed in that document. Indeed this document does not contain a general disclosure of using a phospholipid-disrupting agent in animal feed.

Other documents that refer to antimicrobial effects for more than one component, include a combination of membrane disrupting peptides and lysozyme (Biochemistry 38: 11710 (1999)), combination of lysozyme, hydrogen peroxide and ascorbic acid (J. Bacterial 98:949 (1969)), lysozyme and EDTA, hydrogen peroxide or ascorbic acid (M.J. Physiol. Cell Physiol. 279:799 (2000)).

## Description of the Invention

The present invention provides an animal feed, or an additive or premix composition therefor, comprising two components that show antimicrobial ascinergy. This may allow the improvement of growth and feed conversion ratio of farm, monogastric and/or non-ruminant animals such as pigs. piglets, poultry, (veal) calves and aquatic animals such as fish, and can allow one to reduce the amount of, or omit, an antibiotic as a growth promoter.

The first aspect of the present invention relates to an animal feed composition, comprising:

(a) a compound that produces an antimicrobial (or bacteriostatic or anti-pathogenic) substance; and

(b) a compound that disrupts the (inner and/or outer) phospholipid layer of bacteria.

### *Composition of the Bacterial Cell Wall*

Based on colour formation following staining, bacteria can be divided into two classes, namely Gram positive and Gram negative bacteria. These two classes differ both in the composition and the structure of their cell walls. The major component for both Gram positive and Gram negative bacteria cell walls is peptidoglycan. The peptidoglycan

is a thick rigid layer consisting of an overlapping lattice of two sugars that are cross-linked by amino acid bridges. The exact molecular structure of this layer is species specific.

The two sugars are N-acetyl glucosamine and N-acetylmuramic acid, which are linked through a ß-1,4-glycoside bond. Attached to N-acetylmuramic acid, which is a compound uniquely found in bacterial cell walls, is a side chain usually consisting of four amino acids. The most commonly found amino acids are L-alanine, D-alanine, D-glutamic acid, and a di-basic amino acid, usually diaminopimelic acid. The N-acetylglucosamine, N-acetylmuramic acid and the amino acid side chain forms a single peptidoglycan unit that can link with other units via covalent bonds to form a repeating polymer.

The polymer is further strengthened by crosslinks between the third amino acid (D-glutamic acid) of one unit and the fourth amino acid (diaminopimelic acid) of the next glycan tetrapeptide. The linker peptide of some bacteria contain glycine, serine and threonine. The degree of crosslinking determines the degree of rigidity. Peptidoglycan can be thought of as a strong woven mesh that holds the cell shape. It is not a barrier to solids, since the openings in the mesh are large enough for most type of molecules to pass through them.

The cell walls of Gram positive bacteria consist almost entirely of the peptidoglycan layer, which forms a heavy crosslinked woven structure that wraps around the cell. It is very thick with peptidoglycan accounting for about 50% of the weight of the cell, and 90% of the weight of the cell wall. It is about 20 to 80nm thick.

The cell walls of Gram negative bacteria contain markedly less peptidoglycan, only 15 to 20% of the cell wall being made up of peptidoglycan, and this is only intermittently crosslinked.

Gram negative bacteria further differ from their Gram positive counterparts in that their cell wall contains an extra lipid layer. This is the phospholipid layer or outer membrane. This outer membrane, which is made of a lipopolysaccharide layer, encloses the periplasmic space. The lipid portion of this layer contains lipid A, a toxic compound that is responsible for most of the pathogenic effects associated with harmful Gram negative bacteria.

*Compound or enzyme that produces an antimicrobial substance*

The antimicrobial substance (which may be a bacteriostatic, growth-retarding or antipathogenic substance) may result from catalysis or reaction by an enzyme. The

substance may therefore be a reaction product. The substance is suitably toxic to bacteria, such as Gram positive or Gram negative bacteria.

The substance may be phosphatidyl choline (PC), hydrogen peroxide ($H_2O_2$), HOCl, hypothiocyanate (OSCN), nitric oxide and/or xylitol-5-phosphate.

The compound may be a protein, e.g. an enzyme, such as a (phospho) lipase, oxidase, peroxidase, lipoxygenase, synthase and/or phosphorylase.

The enzyme may be an oxidase, in which case the toxic compound may be $H_2O_2$, HOCl and/or OSCN. Suitable oxidases include glucose oxidase, galactose oxidase, hexose oxidase, methanol oxidase, xanthine oxidase, sulphydryl oxidase, NADPH oxidase, nitroalkane oxidase or a (e.g. poly) phenyl oxidase (such as catchol oxidase or tyrosinase), monoamine oxidase, copper amine oxidase or cytochrome oxidase. The enzyme may be a peroxidase (such as horseradish peroxidase, chlorobromo peroxidase, lacto peroxidase), a lipoxygenase, superoxide dismutase or a nitric oxide (NO) synthase (which generates nitric oxide).

The enzyme may be one that generates an aldehyde, such as carboxylic acid reductase, aldehyde oxidoreductase, alcohol oxidase or methanol oxidase.

The enzyme may be naturally occurring or may be produced recombinantly. The enzyme may have been produced by expression of a heterologous gene in a microorganism, for example in *Kluyveromyces lactis* or *Aspergillus*. The enzyme may be present as an inactive pro-form that can be activated on ingestion, for example in the GI tract, suitably by a proteolytic processing.

The amount of the enzyme is preferably such that is is effective, e.g. antimicrobial, or that it can produce enough antimicrobial substance to kill microbes (e.g. bacteria).

A preferred oxidase is glucose oxidase. This enzyme may be present at a concentration to give from 10 to 10,000 , preferably from 25 or 100 to 1,500 or 5,000, and more preferably from 50 or 200 to 1,000 or 2,500 Sarrett U per kilogram (or unit) of animal feed. Thus preferably the enzyme may be present at an amount, by weight, to give a final concentration in the animal feed of from 0.05 to 50 milligrams of protein per kg of feed, preferably from 0.08 or 0.13 to 7.5 or 25 milligrams of protein per kg of feed, and more preferably from 0.25 or 0.5 to 5.0 or 10 milligrams of protein per kg of feed, for example for *A. niger*-derived glucose oxidase.

*Compounds that disrupt the phospholipid layer*

Such a compound may disrupt the inner and/or (preferably) the outer phospholipid layer. It may be a protein, such as an enzyme, or an inorganic compound. The compound may be a surfactant or detergent, a chelating agent or an insect glycopeptide.

The amount of the compound may be an effective amount, e.g. antimicrobial, or that it can disrupt the phospholipid layer (of bacteria).

Suitable compounds include polyunsaturated fatty acids (PUFAs). A PUFA may be present in the animal feed at no more than 100 g, such as no more than 10 g, preferably no more than 1 g per kg of animal feed. Even lower concentrations of PUFA may be used, for example at least 0.0001g, such as at least 0.001g, preferably at least 0.002g per kg of feed. Suitable amounts are from 0.1 to 0.0001 g of PUFA per kg of feed, preferably from 0.02 or 0.05 to 0.002 g of PUFA per kg of feed and more preferably from 0.01 to 0.004 g of PUFA per kg of feed.

These amounts refer to the weight of the PUFA, and so if the PUFA is added in the form of an oil (e.g. having for example from 30 to 40% of the PUFA), then the amount of oil present (or added) can be calculated accordingly, for example by multiplying the amount of the PUFA by 100/X where X is the percentage of the PUFA in the oil. Hence, for example with a 30 or 35 to 40, 45 or 50% PUFA content, the amount of oil that can be added may vary proportionally, such as from 0.33 or 0.25 down to 0.00033 or 0.00025g of oil per kg of feed. Other amounts and intermediate ranges can be calculated on the same basis, starting with the figures for the PUFA in the previous paragraph.

The PUFA may be an $\Omega$-3 or an $\Omega$-6 PUFA. Preferably it is present in an oil, e.g. an edible oil. The oil may be microbial or single cell oil or a vegetable oil. Preferably the PUFA is in a liquid form, such as will occur if it is present in an oil. Preferred PUFAs are $C_{18}$, $C_{20}$ or $C_{22}$ ($\Omega$-6 or $\Omega$-3) PUFAs.

Preferred $\Omega$3 and $\Omega$6 PUFAs include:

($\Omega$3) docosahexaenoic acid (DHA, 22:6 $\Omega$3), suitably from algae or fungi, such as the (dinoflagellate) Crypthecodinium or the fungus Thraustochytrium;

($\Omega$6) $\gamma$-linolenic acid (GLA, 18:3 $\Omega$6);

($\Omega$3) $\alpha$-linolenic acid (ALA, 18:3 $\Omega$3);

conjugated linoleic acid (CLA, octadecadienoic acid);

($\Omega$6) dihomo-$\gamma$-linolenic acid (DGLA, 20:3 $\Omega$6);

($\Omega$6) arachidonic acid (ARA, 20:4 $\Omega$6); and

($\Omega3$) eicosapentaenoic acid (EPA, 20:5 $\Omega3$).

The microbial oil may thus comprise an $\Omega3$ or an $\Omega6$ PUFA. The $\Omega3$ PUFA (e.g. DHA)-containing oil may be a marine, e.g. fish (such as tuna) oil. The $\Omega6$ and/or $\Omega3$ PUFA (e.g. ARA, DHA or EPA)-containing oil can be a microbial or single cell oil.

An $\Omega6$ and/or $\Omega3$ PUFA-containing microbial oil (e.g. GLA, ARA and EPA) can be obtained from fungi, such as *Mortierella*, *Pythium* or *Entomophthora*. $\Omega3$ PUFAs (e.g. EPA) can be produced from algae such as *Porphyridium* or *Nitzschia*.

Preferably the microbial (or $\Omega6$ or $\Omega3$ (e.g. ARA, DHA or EPA containing)) oil can be produced by a single cell or a microorganism. This may be a bacteria, yeast, algae or fungi. Preferred fungi are of the order *Mucorales*. The fungus may be of the genus *Mortierella*, *Phycomyces*, *Blakeslea* or *Aspergillus*. Preferred fungi are of the species *Mortierella alpina*, *Blakeslea trispora* and *Aspergillus terreus*.

Preferred yeasts are of the genus *Pichia* or *Saccharomyces*, for example *Pichia ciferrii*. Bacteria can be of the genus *Propionibacterium*. Preferred algae are dinoflagellate and/or belong to the genus *Crypthecodinium*, for example are of the species *Crypthecodinium cohnii*.

The $\Omega6$ and/or $\Omega3$ PUFA-containing oil may be an edible oil or a vegetable oil. These include blackcurrant, borage and primrose oils, and often contain an $\Omega6$ PUFA, e.g. GLA. They also include olive, sunflower and soybean, soy flower oils, for example cooking and/or salad oils.

The PUFA may be in the free fatty acid form, or an ester form, such as as a triglyceride. If it is present in an oil, then preferably at least 50%, such as at least 60%, or at least 70%, of the PUFA is in triglyceride form. However, the amount of triglyceride may be higher, such as at least 85%, 90% or at least 95% of the oil. Of these triglycerides, preferably at least 40%, 50% and optimally at least 60% of the PUFA is present at the $\alpha$-position of the glycerol (present in the triglyceride backbone), also known as the 1 or 3 position.

The PUFA can be in the form of a free fatty acid, as a fatty acid ester (e.g. a methyl or ethyl ester), as a phosophilipid and/or in the form of a triglyceride. Preferred PUFAs include ARA, DHA, EPA and/or GLA. Of these, ARA is preferred.

The PUFA may be from a natural, such as vegetable oil or marine, source or may be derived from a single cell or microbial source. The PUFA may be produced by a bacteria, fungus or yeast. The fungi are preferred, preferably of the order *Mucorales*, for example

*Mortierella*, *Pythium* or *Entomophthora*. Preferred source of ARA is *Mortierella alpina* or *Pythium insidiosum*.

The PUFA may act by being a surfactant. Other non-PUFA surfactants are contemplated, including tri-n-butylphosphate, cetyl pyridinium chloride and/or glycerol mono-oleate.

In some embodiments it is preferred that the or each compound (e.g. PUFA) is still present inside the microorganism (that produced it). Hence the compound may be added as microorganism cells, such as biomass. The cells may be mixed with the animal feed (or with one or more feed substance(s) or ingredients). The microorganism may produce one or more of the two types of compounds.

In a typical PUFA production (by fermentation) process the amount of PUFA produced may be from 7 to 10g/kg broth (i.e. wet biomass). Hence the amount of broth (we cells) to be added, or present in, the feed composition can be calculated by multiplying the amount of PUFA desired by a factor of 70 or 100 (e.g. 10g broth/kg feed gives a PUFA concentration of 0.1g/kg feed). If a dried biomass is added or used instead, then the dired cells can have a PUFA amount of 100 to 200, such as 140 to 180g/kg cells, and so to obtain the amount of PUFA one multiplies the amount of PUFA by 10 or 20 to give the amount of dried cells per kg feed.

The phospholipid-disrupting layer compound may be a peptide, such as a glycopeptide or a thiopeptide. The compound may thus be a cecropin, defencin, attacin, melittin, proline rich peptide, diptericin, pleurocidin, trichogin, alexomycin or nisin.

The compound may be a chelating agent, for example one bearing a negative charge. It may therefore chelate a positive ion, for example of valency II. This may include alkaline earth metals, such as calcium and magnesium. A suitable chelating agents include EDTA (ethylene diamine tetra acetic acid), CDTA, HDTA, NTA and/or IDA.

Other compounds include bile salts (such as deoxycholate, organic or inorganic acids (lactic acid, hydrochloric acid, ascorbic acid), other enzymes (rhodanese), imidazols (such as miconazol), or cinnamon aldehyde.

A further example of a phopholipid disrupting compound is phospholipase $A_2$ ($PLA_2$). This may be from a mammalian, such as a human, source. The use of this enzyme in animal feeds is already known (EP-A-0,743,017). Other phospholipases may be used, and these include phospholipase $A_1$ (EC 3.1.1.32), phospholipase B (lysophospholipase), phospholipase C and phospholipase D. Phospholipase $A_2$ has the activity EC 3.1.1.4.

The phospholipase may be from a natural source, or it may be produced recombinantly, for example by expression of an heterologous gene in a microorganism, for example in *Kluveromyces lactis* or *Aspergillus*. The phospholipase may be present as an inactive pro-form that can be activated on ingestion, for example in the GI tract, suitably by proteolytic processing.

The phospholipase may be added to a feed at a concentration which varies according to the type of phospholipase employed, and the target animal. However, as a guidline, the concentration of a phospholipase is from 1,000 to 5 million IU (International Units) per kg of phospholipid, such as from 10,000 to 500,000. The definition of the International Unit for phospholipase activity can be found in Example 1 of EP-A-0,743,017.

Thus, preferably the enzyme (or compound) may be present at an amount, by weight, to give a final concentration in the animal feed of from 0.005 to 5 (or 10 or 20) milligrams per kg of feed, preferably from 0.01 or 0.025 to 2.5 or 4 milligrams per kg of feed, and more preferably from 0.05 or 0.1 to 1.0 or 0.7 milligrams per kg of feed, for example in the case of pig pancreas $PLA_2$ produced in *A. niger*.

In the animal feed, the concentration of phospholipid may be from 0.5 g to 10.0 g per kg of feed. Consequently, the phospholipase may be present in a range of from 0.5 to 50,000 IU per kg feed, preferably from 5,0 to 5,000 IU per kg of feed. Of course, the dosage of phospholipase can be adjusted, if the phospholipid content of the feed is outside this range, or is not present at all.

Preferably the phospholipase is $PLA_2$. This has been expressed in various organisms, such as *E. coli*, *Saccharomyes cerevisiae* and *Aspergillus niger*. It is therefore expected successful (e.g. heterologous) expression of this phospholipase can be obtained in a wide range of microorganisms.

If the animal feed comprises a phospholipid, it is preferably a lecithin. If $PLA_2$ is used, then preferably this is from a mammalian source, such as from a bovine, porcine, murine rat or human source.

*Bacteria*

As mentioned before, the animal feeds of the present invention are active against bacteria, in particular Gram negative bacteria, as these have both the lipid/lipopolysaccharide layer as well as the peptidoglycan layer. A list of Gram negative

bacteria which the compounds can be synergistically active against in the present invention is provided below.

| Class | Genera | Species |
|---|---|---|
| Acetobacteriaceae | Acetobacter, Gluconobacter, Frateuria | *A. aceti* |
| Alcaligenaceae | Alcaligenes, Deleya, Achromobacter | *A. faecalis* |
| Bacteroidaceae | Bacteroides, Porphyromonas, Fusobacterium, Leptotrichia and Selenomonas | *S. rumantium* |
| Chromatiaceae | Ameobobacter, Chromatium, Lamprobacter Lamprocystis, Thiocapsa, Thyocystis Thiodictyon, Thiopedia, Thiospirillum | *C. okenii* |
| Enterobacteriaceae | Escherichia, Salmonella, Shigella, Erwinia, Enterobacter, Serratia | *E. coli* |
| Legionellaceae | Legionella | *L. pneumophila* |
| Neisseriaceae | Neisseria, Kingella, Eikenella, Simonsiella Alysiella | *N. gonorrheae* |
| Nitrobacteriaceae | Nitrobacter, Nitrospena, Nitrococus, Nitrosipra | *N. winogradskyi* |
| Pseudomonadaceae | Pseudomonas, Xanthomonas, Zoogloea, Fraturia | *P. aruginosa* |
| Rhizobiaceae | Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium | *R.laguminosarum* |
| Rickettsiaceae | Rickettsia, Rochalimae, Ehrlichia, Cowdria Neorickettsia | |
| Spirochaetaceae | Triponema, Borrelia | *T. pallidum* |
| Vibrionaceae | Vibrio, Aeromonas, Plesiomonas and Photobacterium | *V. cholerae* |

Of these Gram negative bacteria, preferred are those of the class Vibrio, Neisseria, or Salmonella.

Although particularly effective against Gram negative bacteria, the compounds used in the invention can also be active against Gram positive bacteria. Suitable Gram positive bacteria are listed below.

| Class | Genera | Species |
|---|---|---|
| Bacillaceae | Bacillus, Sporolactobacillus<br>Sporocarcina, Filibacter, Cayophanum<br>Clostridium | *B. botulinum*<br>*B. cereus,*<br>*B. coagulans*<br>*B. mycroides*<br>*B. pumilis*<br>*B. subtilis*<br>*B. thuringiensis* |
| Micrococcaceae | Arthrobacter and Micrococcus | *M. luteus,*<br>*M. roseus,*<br>*M. lysoeiktus,*<br>*M. radiochrans* |
| Peptococcaceae | Peptococcus, Peptostreptococcus<br>Ruminococcuss, Sarcina, Coprococcus | *P. niger* |

Preferably the Gram positive bacteria are of the group *Cornynebacterium, Propionibacterium*, *Clostridium*, *Lactobacillus* and/or *Bifidobacterium*.

*Animal Feed Compositions*

The compounds, if enzymes, can be produced on industrial scale and/or may be recombinant. The enzyme may be naturally occurring or may be a (e.g. recombinant) variant or mutant thereof.

The compound is preferably recombinantly produced such as by expression of a heterologous gene or cDNA in a suitable organism, or alternatively by homologous (over)expression of a suitable endogenous gene. The glucose oxidase gene, for example, has been overexpressed in recombinant systems (WO-A- 89/12675, Chiron). Enzymes can be recombinantly expressed by expression of the gene in *Aspergillus niger* (Archer, D.B. *et al.*, Bio/Technology 8: 741-745 (1990)). An enzyme mutant (produced by protein engineering) can also be used which may have better heat stability and/or stronger antimicrobial action.

A second aspect of the invention relates to a premix or additive composition to be added to one or more edible feed substance(s) or ingredient(s), for example to prepare or for supplementation feed composition (of the first aspect). This can comprise the two layer-disrupting compounds. Preferably the additive or premix comprises from 10 to 1000,

such as from 25 or 50 to 750, preferably from 75 or 100 to 250 or 500, times as much of either of the two compounds as the feed. This is because the premix can be "diluted" by a factor of 10 to 1,000 (so that the premix constitutes 10% to 0.1% of final feed) when making the animal feed. This premix may be in the form of granules or pellets.

A third aspect of the invention relates to a process for the preparation of an animal feed composition, the process comprising adding to (or supplementing) an animal feed, or to one or more edible feed substance(s) or ingredient(s), the two layer-disrupting compounds.

The or each compound can be added to the animal feed composition separately from any feed substance(s) or ingredient(s), individually or in combination of other feed additives. Alternatively or in addition the compound can be in integral part of one of the food substances. The invention includes both preparing a feed composition with the two compounds or supplementing an existing feed composition with these two compounds.

A preferred method for the addition of the compound to the animal feed is to add the compound as transgenic plant material and/or (e.g. transgenic) seed. This is particularly suitable if the compound is protein, such as an enzyme. The compound may be synthesised through heterologous gene expression, for example the gene encoding the desired enzyme may be cloned into a plant expression vector, under control of the appropriate plant expression signals, for example a tissue specific promoter, such as a seed specific promoter. The expression vector containing the gene including the enzyme can be subsequently transformed into plant cells. Transformed cells can be selected for regeneration into plants. The thus obtained transgenic plant can be grown and harvested.

Those parts of the plants containing the heterologous (plant) protein can be included in one of the compositions of the invention, either as such or after further processing. Reference here is made to WO-A-91/14772, which discloses general methods for the (heterologous) expression of enzymes in (transgenic) plants. This includes methods for seed-specific expression of enzymes. The compound, such as the protein, may be contained in the seed of the transgenic plant. It may also however be contained in other plant parts such as roots, stems, leaves, wood, flowers, bark and/or fruit.

The addition of the compound in the form of a transgenic plant material, such as transgenic seed, may require the processing of the plant materials such as to make the compound available, or at least to improve the compounds availability. Such processing techniques may include various mechanical techniques, such as milling and/or grinding, or thermomechanical treatments, such as extrusion or expansion.

*Exclusions*

Preferably the animal feed of the invention does not contain any antibiotics. It may be free of a (supplementary or added) mineral component (such as zinc and/or iodine) and/or an immunomodulating agent (such as ascorbic acid). The composition may not include a combination of lysozyme, glucose oxidase and arachidonic acid or lysozyme, glucose, glucose oxidase and ascorbic acid. Other excluded compositions may include those comprising a combination of $PLA_2$ and lysostaphin, ascorbic acid and lysozyme.

*Production of compounds by microorganisms*

Although one or more of the compounds can be produced by a microorganism, for many situations (the producing) micro-organisms will not be added to or present in the feed, or at least live (or viable) organisms, such as bacteria, are not present in the feed. Hence in this case the composition is free from any microorganisms that produced one or more of these compounds (or micro-organisms from *Streptomyces*). Furthermore, the composition may be devoid of micro-organisms that produce lactic acid inside the animal (e.g. those of the genus *Lactobacillus* or *Enterococcus*). Typically, before addition of the compounds, the feed composition will be heated to kill, or reduce the number of, any bacteria present in the feed.

*Uses of animal feed*

A fourth aspect of the invention relates to a process for promoting growth, feed conversion or antibacterial activity, in a monogastric or non-ruminant animal, the process feeding the animal a compound that produces an antimicrobial substance and a compound that disrupts the phosopholipid layer bacteria. The animal can be fed the animal feed of the first aspect or feed preparable by the third aspect.

Suitable animals include farm, monogastric and/or non-ruminant animals such as pigs (or piglets), poultry (such as chickens and turkeys), calves, veal calves or aquatic (e.g. marine) animals, for example fish.

A further aspect relates to the use of a composition of the second aspect as an additive for a monogastric or non-ruminant animal feed composition.

The compositions of the invention may be active *in vivo* (e.g. not *in vitro*), or only once ingested or inside the animal. The compounds may thus not be effective since the compositions may be too dry, e.g. they have a water content of no more than 10, 20, 30, 40

or 50%. Once ingested and inside the animal (e.g. in the stomach or rumen) there may be sufficient liquid (or water) for the compounds to become active or effective (e.g. antimicrobial, or layer disrupting).

*Animal Feed Components*

The compositions of the invention, in particular additive or premix compositions, can be either in liquid or solid form. If a solid, then this may be a powder, a granulate, extrudate or it may be pellets. For a solid form, the amount of water present may be below 20, 15 or even 10%, such as from 2 to 10%, 3 to 8% or 4 to 7%. The or each compound (e.g. enzyme) may be present at from 1 to 30%, such as 2 to 20%, for example 3 to 15%, and optimally at from 4 to 14% (on a dry matter basis). The remainder may comprise carbohydrates and/or carbohydrate polymers (such as starch and/or modified starch), for example at least 70, 80, 90 or 95%, such as from 75 to 90%. The composition may have a coating, for example if it is in a pellet, granulate, or extrudate form. There may thus be one or more coats on the outside of the composition, comprising one or more coating materials. If present, the coating (or coating materials) may be present at from 1 to 10%, such as from 2 to 6%, optimally at from 3 to 5%. The composition may have one or more stabilisers (such as glycerol and/or sorbitol) and/or one or more preservatives (such as sorbate and/or benzoate).

If the composition is a liquid, then the water (or moisture) content will be higher. The water content may be up to 40, 50 or 60%, for example from 25 to 65%, optimally from 35 to 55%. If a stabiliser is present, this may be at an amount of from 45 to 65%, such as from 50 to 60%, optimally from 52 to 58%. The stabiliser is preferably sorbitol and/or glycerol.

A description of the preparation of pellets and granules, in particular carbohydrate based enzyme granulates, is described in WO-A-98/54980 (International Application No. PCT/EP98/03327), the contents of which is incorporated by reference.

The composition may comprise a carrier which may comprise at least 15% of an edible carbohydrate polymer. The carrier may be in particulate or powder form. However, if the composition is a liquid, it may be in the form of a solution or a slurry. The polymer preferably comprises glucose, or glucose-containing units, although it can contain glucopyranose units, amylose and/or amylopeptin. In addition, or instead of starch, a glucan, peptin or glycogen can be used. Preferably at least 15%, such as at least 30%, at

least 40%, for example at least 60%, optimally at least 80% of the composition (or the solid carrier) comprises the carbohydrate polymer.

Additional details of enzyme-containing compositions for animal feed can be found in

WO-A-98/55599 (International Application No. PCT/EP98/03328), the contents of which is also incorporated by reference. Although this document primarily deals with phytases, its teachings are equally applicable to other compounds, in particular enzymes.

Animal feed compositions of the first aspect will usually contain one or more feed ingredients or substances. These are ingredients and substances intended for consumption by an animal, and is therefore in a form suitable for ingestion and nutrition for an animal. This will therefore usually exclude human foodstuffs, or food substances or ingredients intended or destined for consumption by humans. Preferably the feed composition is both edible and digestible by the animal.

Suitably the substances and/or ingredients have a dry matter content of at least 80, 85, 90 or 95%. The protein content of the composition (or the substances and/or ingredients) may vary considerably, but may be from 5 to 20%, such as 10 to 15%, for example vegetable and/or plant products or parts thereof, such as buckwheat, rice, wheat, barley or corn. Substances or ingredients with higher protein contents, such as from 45 to 95%, e.g. 50 to 80%, may be provided, for example peanuts, poultry feathers, soy bean (or products thereof), sunflower (e.g. seeds) or casein. Preferred animal feed compositions may therefore comprise one or more of oats, pea (seeds), peanuts, soy beans, sunflower, canola, casein, coconut, corn, meat, millet, potato, rice, safflower and/or wheat. Preferably the composition (and substances or ingredients) have a crude fibre content below 30%, 25%, 20%, 15% or even below 10%. Similarly, the calcium content may be below 2%, such as 1%, below 0.5% and preferably less than 0.2%. The total phosphorous content of the (animal feed composition) is preferably from 2 to 0.01%, such as from 1 to 0.1%, optimally less than 0.5%.

The precise substances and ingredients can vary depending on the animal to be fed. An alternative composition may comprise one or more of bakery waste, sugar beet, brewers grain, canola, cassava, corn, fababean, fish (such as anchovy or herring meal), lentils, meat and/or millet.

Preferred features and characteristics of one aspect of the present invention are applicable to another aspect *mutatis mutandis*.

The present invention will now be described by way of example with reference to the following Examples, which are provided by way of illustration, and are not intended to limit its scope.

## EXAMPLES.

Characterization of antimicrobial compounds

Glucose oxidase (EC 1.1.3.4), an oxidase capable of generating hydrogen peroxide, was obtained as a commercial product under the trade mark FERMIZYME GO™ 1500 from DSM Food Specialties, PO Box 1, 2600 MA DELFT, The Netherlands. This enzyme preparation exhibits an activity of 1500 Sarrett Units per gram. One Sarrett unit is the amount of enzyme that will cause an uptake of $10mm^3$ of oxygen per minute in a Warburg manometer at 30°C in the presence of excess oxygen and 3.3% glucose monohydrate in a phosphate buffer with a pH of 5.9. The enzyme was produced by the fungus *Aspergillus*.

Phospholipase $A_2$ was obtained through production of pig pancrease $PLA_2$ in *Aspergillus niger* from DSM Food Specialties, Agri Ingredients, P.O. Box 1 , 2600 MA Delft, The Netherlands. This process is described in WO-A-96/36244. Phospholipase concentrations are defined by so-called Egg Yolk Units (EYU). One EYU is defined as the amount of phospholipase enzyme that releases 1 μmol of acid per minute from egg lecithin at pH 8 and 40°C.

Arachidonic acid (ARA) was obtained from DSM/ Food Specialties, Agri Ingredients, P.O. Box 1, 2600 MA Delft, The Netherlands under the trade mark VEVODAR™. This is a microbial oil (ARA content at least 35%) obtained by culturing the fungus *Mortierella alpina*.

Comparative Examples 1 to 3 and Example 4

Application of a combination of a compound that produces a toxic antimicrobial substance (glucose oxidase) and a compound that disrupts the phospholipd layer in animal feed for poultry

Trials we carried out with broilers to test the efficacy of glucose oxidase and phospholipase alone or in combination. Male and female broilers (Ross) were used in this trial; the animals were sexed and housed separated (4 pens per sex per treatment). Upon arrival, animals we weighed, and divided into floorpens equalising the average weights and its deviation between treatments. Fifteen animals were kept per pen. The pens were situated

in an artificially heated, ventilated and illuminated broiler house. The floor space of each pen was 0.75 $m^2$; wood shavings were used as bedding material. The broiler house was illuminated for 23 hours per day. During the experimental period, light intensity was gradually reduced. The temperature was gradually reduced from 33°C the first day to 21°C at day 28. The animals had been vaccinated against New Castle disease and Infectious Bronchitis. The experiment lasted 28 days.

The experimental diets were offered *ad lib.* to the animals. Water was freely available. The feed was pelleted (with temperatures below 65°C) at a diameter of 2.5 millimeter.

The experiment comprised the following treatments:

1) basal diet (negative control)
2) basal diet + glucose oxidase (Gox; 500 Sarrett units/kg of feed)
3) basal diet + phospholipase $A_2$ ($PLA_2$; 500 EYU/kg of feed.
4) basal diet + glucose oxidase (500 Sarrett units/kg of feed) + phospholipase $A_2$ (500 EYU/kg of feed).

Gain and feed conversion were measured. The composition of the feed (basal diets) used was:

| Ingredients | Content (%) |
|---|---|
| Rye | 10 |
| Wheat | 41.85 |
| Soy oil | 2 |
| blended animal fat | 6 |
| Rape seed meal | 7.5 |
| Soya bean meal (45.4% crude protein) | 18.5 |
| Full fat toasted soya beans | 5 |
| Soya isolate | 2.5 |
| Corn gluten meal | 2.5 |
| Vitamins/premix | 1 |
| Limestone | 1.4 |
| Monocalciumphosphate | 1.2 |
| Salt (NaCl) | 0.25 |
| L-lysine.HCL | 0.14 |
| DL-methionine | 0.16 |
| ME broilers (MJ/kg) | 12.02 |

| | |
|---|---|
| Crude protein (%) | 22.4 |
| Crude fat (%) | 10.3 |
| Lysine (digestible, %) | 1.06 |
| Methionine + cystine (digestible, %) | 0.78 |

The glucose oxidase and phospholipase were added to this basal diet by mixing them first with a carrier.

The effects of the glucose oxidase and phospholipase on growth and feed conversion ratio in broilers after 28 days are shown below in Table 1.

TABLE 1

| Example | Diet | Feed Intake (g) | Growth (g) | Feed conversion ratio |
|---|---|---|---|---|
| 1 | Basal diet | 2173 | 1319 | 1.65 |
| 2 | Basal diet + glucose oxidase | 2113 | 1308 | 1.62 |
| 3 | Basal diet + phospholipase | 2159 | 1347 | 1.60 |
| 4 | Basal diet + glucose oxidase + phospholipase | 2087 | 1352 | 1.54 |

Both compounds improved performance, but the combination showed a synergistic performance.

Comparative Examples 5 to 7 and Example 8

Application of a combination of a compound that produces a toxic antimicrobial substance (glucose oxidase) and a compound that disrupts the phospholipd layer in animal feed for piglets

Crossbred piglets (equal number of barrows and gilts; in total 80 animals) of a similar age and weight were used in this trial. They were housed in environmentally controlled rooms, and had *ad lib.* access to feed and water at all times. Temperature, ventilation and illumination were applied according to common practice. The piglets were allotted to one of four treatments. There were two piglets in each pen with 10 replications

(weight blocks) per treatment. The sexes were divided evenly over the treatments. The trial started 2 weeks after weaning of the piglets, at an age of approximately 38 days, and lasted for 6 weeks.

Body weight and pen feed consumption were measured after three and after six weeks of the experiment.

The basal diet was a typical corn-soybean meal diet, with the following composition:

| Raw Material | Content (%) |
|---|---|
| Corn | 63.4 |
| Soyabean meal | 33.73 |
| Dicalcium phosphate | 1.29 |
| Limestone | 0.74 |
| Salt (NaCl) | 0.33 |
| L-lysine.HCl | 0.01 |
| Vitamin-trace mineral premix | 0.5 |

The diet was calculated to contain 3265 kcal ME/kg, 20.5% crude protein, 1.15% lysine and 0.68% methionine + cystine. No antibiotic was added to the feed.

The experiment comprised the following treatments (Examples 5 to 8):

5) basal diet (negative control);

6) basal diet + Glucose oxidase (500 Sarrett units/kg of feed)

7) basal diet + arachidonic acid (0.001 g/kg of feed).

8) basal diet + Glucose oxidase ( 500 Sarrett units/kg of feed) + arachidonic acid (0.001 g/kg of feed).

The results obtained in terms of feed intake, growth and feed conversion ratio are shown below in Table 2.

TABLE 2

Average effects of Glucose oxidase and arachidonic acid on growth and feed conversion ratio in piglets (38 to 80 days of age).

| Example | Diet | Daily Feed Intake (g) | Daily gain (g) | Feed Conversion Ratio |
|---|---|---|---|---|
| 5 | Basal diet | 621 | 371 | 1.69 |
| 6 | Basal diet + Glucose oxidase | 603 | 354 | 1.70 |
| 7 | Basal diet + arachidonic acid | 662 | 395 | 1.67 |
| 8 | Basal diet + Glucose oxidase + arachidonic acid | 607 | 382 | 1.59 |

Performance was improved, with the combination showing a synergistic effect.

Example 9: *in vitro* tests

*In vitro* tests were carried out to substantiate the synergistic antimicrobial effect of a combination of an enzyme that can produce a (toxic) antimicrobial substance and a compound that can disrupt the (inner and/or outer) phospholipid layer of bacteria.
In disk diffusion (also called Bauer-Kirby) susceptibility tests, small paper disks (6 mm), impregnated with known amounts of glucose oxidase, arachidonic acid, or phosholipase A2 were placed on the surface of YNB media plates that were inoculated confluently with a standardized suspension of *E. coli* K-12. The glucose oxidase, arachidonic acid, and phosholipase A2, which diffuse into the media, caused a zone of inhibition of growth of *E. coli* around the disk.

The protocol was as follows. A sterile cotton swab was placed in *E. coli* K-12 suspension, and excess fluid was removed by pressing and rotating the cotton against the inside of the tube above the fluid level. The swab was then streaked in at least three directions over the surface of the YNB plate to obtain uniform growth. A final sweep was made around the rim of the plate. The plates were allowed to dry for five minutes. Using sterile forceps, disks containing glucose oxidase, arachidonic acid or phosholipase A2 were applied onto the plates. The plates were incubated within 15 minutes after application of the disks. Following overnight incubation, the diameter of the zone of non-growth was used as a measure of susceptibility.

The paper disks were impregnated in the following solutions:

A; De-ionized water

B: Glucose oxidase (0.5 Sarrett units / ml in deionized water)

C: Arachidonic acid (1 microgram / ml in deionized water)

D: Phospholipase A2 (0.5 egg yolk units / ml in deionized water)

E: Glucose oxidase (0.5 Sarrett units / ml) + arachidonic acid (1 microgram / ml) in deionized water

F: Glucose oxidase (0.5 Sarrett units/ml) + Phospholipase A2 (50 egg yolk units / ml)

The following results were obtained:

| Experiment | zone of inhibited growth (mm) |
|---|---|
| A | 0 |
| B | 7 |
| C | 5 |
| D | 4 |
| E | 19 |
| F | 29 |

## CLAIMS

1. An animal feed composition comprising:

(a) a compound that can produce an antimicrobial substance; and

(b) a compound that disrupts the phospholipid layer of bacteria.

2. An animal feed additive or premix composition comprising:

(a) a compound that can produce an antimicrobial substance; and

(b) a compound that disrupts the phospholipid layer of bacteria.

3. A composition according to claim 1 or 2 wherein the antimicrobial substance is toxic to Gram negative or Gram positive bacteria.

4. A composition according to any one of the preceding claims wherein the substance comprises phosphatidyl choline (PC), hydrogen peroxide ($H_2O_2$), HOCl, hypothiocyanate (OSCN), nitric oxide (NO) or an aldehyde.

5. A composition according to claim 5 wherein the compound that produces an antimicrobial substance is an enzyme comprising a (phospho)lipase, oxidase, peroxidase, lipoxygenase, synthase and/or phosphorylase.

6. A composition according to claim 5 wherein the enzyme comprises glucose oxidase, galactose oxidase, hexose oxidase, methanol oxidase, xanthine oxidase, sulphydryl oxidase, NADPH oxidase, nitroalkane oxidase, phenyl oxidase, monoamine oxidase, copper amine oxidase and/or cytochrome oxidase.

7. A composition according to claim 5 wherein the enzyme comprises a peroxidase which is horseradish peroxidase, chlorobromo peroxidase, or lacto peroxidase.

8. A composition according to claim 5 wherein the enzyme comprises lipoxygenase, nitric oxide synthase, xylitol phosphorylase, carboxylic acid reductase or aldehyde oxidoreductase.

9. A composition according to claim 5 wherein the enzyme comprises glucose oxidase and is at a concentration of from 10 - 10,000 Sarrett units per kg of animal feed.

10. A composition according to claim 5 wherein the enzyme comprises glucose oxidase at a concentration of from 0.05 – 50 milligrams per kg of animal feed.

11. A composition according to any preceding claim wherein the phospholipid layer disrupting compound comprises a protein, such as an enzyme, or an organic or inorganic compound.

12. A composition according to claim 11 wherein the compound comprises a (phospho)lipase, a polyunsaturated fatty acid (PUFA), or a chelating agent.

13. A composition according to claim 12 wherein the (phospho)lipase is from a mammalian, e.g. human source and/or is $PLA_2$.

14. A composition according to claim 12 wherein the enzyme comprises phospholipase and is at a concentration of from 5 to 5,000 Egg Yolk Units per kg of animal feed.

15. A composition according to claim 12 wherein the enzyme comprises phospholipase and is at a concentration of from 0.005 to 5 milligrams per kg of animal feed.

16. A composition according to any of claims 11 to 15 wherein the phospholipid layer disrupting compound is derived from an animal, an animal product, plant, plant product, algae, algal product, single cell (product) or microorganism.

17. A composition according to claim 16 wherein the compound is of animal, plant, algal or microbial origin and/or is recombinant.

18. A composition according to any of claims 12 to 17 wherein the PUFA comprises an $\Omega$-3 or $\Omega$-6 $C_{18}$, $C_{20}$ or $C_{22}$ PUFA.

19. A composition according to claim 12 wherein the PUFA is in the form of free fatty acid, salt, fatty acid ester, phospholipid or mono-, di- or triglyceride.

20. A composition according to claim 12 wherein the PUFA comprises arachidonic acid (ARA), optionally at a concentration of from 0.0001 to 100 grams per kg of animal feed

21. A process for the preparation of a feed composition, suitable for a monogastric or non-ruminant animal, the process comprising adding a compound that produces an antimicrobial substance and a compound that disrupts the phospholipid layer of bacteria to an animal feed, or mixing a feed additive or premix composition according to any of claims 2 to 18, with one or more edible feed substance(s) or ingredient(s).

22. An animal feed composition comprising an additive or premix composition according to claim 2 and one or more edible feed substance(s) or ingredient(s).

23. A process for promoting growth and/or feed conversion in a monogastric or non-ruminant animal, the process comprising feeding the animal compound that produces an antimicrobial substance and a compound that disrupts the phospholipid layer bacteria or a composition as defined in any of claims 1 to 20.

24. A process according to claim 23 where the animal is a pig, piglet, poultry, veal calf or aquatic animal.